ORIGINAL ARTICLE

Black reefs: iron-induced phase shifts on coral reefs

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The Line Islands are calcium carbonate coral reef platforms located in iron-poor regions of the central Pacific. Natural terrestrial run-off of iron is non-existent and aerial deposition is extremely low. However, a number of ship groundings have occurred on these atolls. The reefs surrounding the shipwreck debris are characterized by high benthic cover of turf algae, macroalgae, cyanobacterial mats and corallimorphs, as well as particulate-laden, cloudy water. These sites also have very low coral and crustose coralline algal cover and are call black reefs because of the dark-colored benthic community and reduced clarity of the overlying water column. Here we use a combination of benthic surveys, chemistry, metagenomics and microcosms to investigate if and how shipwrecks initiate and maintain black reefs. Comparative surveys show that the live coral cover was reduced from 40 to 60% to <10% on black reefs on Millennium, Tabuaeran and Kingman. These three sites are relatively large (>0.75 km²). The phase shift occurs rapidly; the Kingman black reef formed within 3 years of the ship grounding. Iron concentrations in algae tissue from the Millennium black reef site were six times higher than in algae collected from reference sites. Metagenomic sequencing of the Millennium Atoll black reef-associated microbial community was enriched in iron-associated virulence genes and known pathogens. Microcosm experiments showed that corals were killed by black reef rubble through microbial activity. Together these results demonstrate that shipwrecks and their associated iron pose significant threats to coral reefs in iron-limited regions.
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Introduction

The Line Islands (LI) located in the central Pacific harbor several nearly pristine coral reefs (Knowlton and Jackson, 2008). The islands' emergent terrestrial substrates are calcium carbonate reef sands and rubble, devoid of emergent basaltic crust that would release iron into the surrounding waters (Charles and Sandin, 2009). Most of the islands are uninhabited and are isolated from point sources of pollution. Shipwrecks are one exception and have been documented on Kingman, Millennium, Palmyra, Starbuck and Tabuaeran. There are also shipwrecks on similar carbonate atolls throughout the Pacific, including McKean, Canton, Enderbury,

Phoenix and Nikumaroro of the Phoenix Islands, and on Rose Atoll of American Samoa (Sandin et al., 2008; Schroeder et al., 2008; Work et al., 2008). In the LI, the areas surrounding the shipwrecks become black reefs, characterized by high prevalence of fleshy algae—both turf and macroalgae, cyanobacterial mats and corallimorphs, and a dramatic loss of corals and crustose coralline algae (CCA) (Schroeder et al., 2008; Work et al., 2008).

Similar phase shifts in response to shipwrecks where coral- and CCA-dominated communities change to communities dominated by opportunist benthic organisms have been documented on other coral reefs (the term phase shift refers to the observation described by Done (1992), where environmental conditions favor algal overgrowth of previously coral-dominated ecosystems). On Rose Atoll, the percent cover of turf/cyanobacterial assemblages was an order of magnitude higher (>40%) on reefs surrounding the wreck as compared with reference sites on the same island.

This algal community persisted despite the presence of significantly higher abundances of herbivorous fishes at impacted sites (Schroeder *et al.*, 2008). The corallimorph *Rhodactis howesii* has spread over the previously coral-dominated reef terrace on Palmyra Atoll in response to shipwreck debris (Work *et al.*, 2008). High densities of the rapidly proliferating *R. howesii* extended >2 km from the shipwreck, although the abundances progressively declined with distance. Although this work is most pertinent to remote Pacific islands with low iron inputs, coral reefs located on continental shelves may also be susceptible. A bulk carrier, which ran aground on the windward side of Myrmidon Reef, GBR, initiated a phase shift where the red macroalgae *Asparogopsis taxiformis* dominated the benthos and live coral cover was diminished to <1% (Hatcher, 1984). A different circumstance where coral reefs were subjected to iron fertilizations comes from the aftermath of extensive wildfires in Indonesia. Iron enrichment resulting from ash deposited onto the Mentawai reefs located offshore southwest of Sumatra, Indonesia, led to an explosive dinoflagellate bloom that suffocated and killed nearly 100% of corals (Abram *et al.*, 2003). In summary, marine ecosystems occurring in regions with low iron availability can be substantially altered by iron enrichment.

Iron concentrations are extremely limited in the central Pacific ocean (Martin and Fitzwater, 1988), with concentrations ranging from 0.2 to 1 nM (Bruland *et al.*, 1991). Few studies have measured the iron available to the benthic organisms on coral atolls. However, sediments on the reefs consist mostly of calcium carbonate generated *in situ* through the erosion of calcifying organisms and as such unlikely to contain much iron (Entsch *et al.*, 1983). We hypothesize that: (1) iron limits primary production by algae and cyanobacteria on central Pacific coral atolls where there are no emergent basaltic rocks; (2) on these atolls shipwreck-associated iron releases these primary producers from bottom-up controls and (3) the resulting communities of fleshy algae and cyanobacteria either directly compete with the coral or they promote microbes that kill the coral (Smith *et al.*, 2006; Vermeij *et al.*, 2009). In this study we document three black reefs in the LI; measure iron concentrations directly from coral reef algae; investigate the effects of iron enrichment on corals, algae and their associated microbes; and characterize the microbial community associated with a black reef using metagenomics. Together our results support the hypothesis that shipwreck iron is capable of inducing the collapse of reef systems in iron-depleted oceans such as the LI.

Materials and methods

Benthic surveys and sample collection
The benthic community was characterized at 10 m depth on the fore-reef of each island using photoquadrats (Sandin *et al.*, 2008). Two 25-m transect lines were quantified per site and 10 0.72-m² quadrats were assessed along each transect line using digital underwater photographs. Benthic organisms were classified by using Photogrid 1.0; 100 points were randomly chosen and the organism at each point was identified to the lowest taxonomic level possible (genus level for corals, functional group for turfs and crustose coralline algae, and species level for macroalgae).

Algae and rubble samples (mixed algal communities living on carbonate skeletons) were collected at 10 m depth from the shipwreck site on Millennium (April 2009) and from control sites on Millennium and other LI (April 2009 and August–September 2005), and stored in sealed plastic bags at −20 °C on shipboard and at −80 °C in the lab. Voucher specimens of *Derbesia tenuissima* (BISH 743221) from Kingman Reef and *Gelidium isabelae* (BISH 743222) from Millennium Atoll have been archived at the Bishop Museum Herbarium Pacificum, Honolulu, HI, USA.

GIS map of oceanic iron concentrations
The data for oceanic iron concentrations were taken from Behrenfeld *et al.* (2009), wherein concentrations were estimated based on the deposition of soluble iron. Data were imported into the SeaWiFS Data Analysis System (SeaDAS) for image analysis.

Measurement of algal iron concentrations
Algae and rubble samples were decalcified using 3.7 N HCl until carbonate structures were no longer visible and further acid addition produced no bubbling. Remaining tissues were rinsed 3× with 5 ml DI water, dried at 60 °C and weighed on a microbalance (Mettler-Toledo Inc., Columbus, OH, USA). Tissues were then ashed for >12 h at 550 °C and dissolved in nitric acid (HNO$_3$, 0.5 N f/v). Iron concentrations were measured using inductively coupled plasma (Optima 4300 DV ICP-OES 4300; Perkin Elmer, Waltham, MA, USA). Ionized iron atoms were detected by ICP-OES at 259 nm.

The carbon and nitrogen content of algae and rubble tissue was measured using an elemental analyzer (2400 CHN/O EA; Perkin Elmer).

Iron enrichment experiments
Pocillopora meandrina coral samples were collected from a reference site on Tabuaeran Atoll. Individual 3-cm fragments (*n* = 80) were placed in 1 l of reef water in acid-washed Nalgene polycarbonate bottles. Rubble samples were collected from the black reef site on Tabuaeran. Rubble pieces (∼2 cm in diameter) were added to 40 of the aquarium bottles. Eight different treatments, 10 replicates each, were performed: coral, coral + ampicillin, coral + iron,

coral + iron + ampicillin, coral + rubble, coral + rubble + ampicillin, coral + rubble + iron and coral + rubble + iron + ampicillin. For treatments, iron chloride (FeCl$_3$; Sigma, St Louis, MO, USA) was added (1 μM final concentration); ampicillin (Sigma) was added (50 μg ml^{-1} final concentration) at time 0 and refreshed after 48 h. All treatments were incubated at ambient temperature with continuous aeration for 5 days. Coral fragments were photo-documented and the concentration of dissolved oxygen (DO) was measured in each aquarium.

DO measurements
The concentration of DO was measured in each aquarium using a Hach LDO101 Standard DO probe (Hach, Loveland, CO, USA). The aeration tubing was removed from the aquariums ~5 min prior to DO measurement. Measurements were made at the end of the 5-day treatments and approximately 2 h later.

Metagenomic library construction and analysis
At each site, approximately 100 l of seawater were collected from below the boundary layer (in crevices and against the benthos) above ~20 m² of reef by using a modified bilge pump connected to low-density polyethylene collapsible bags (19 l; Cole-Parmer, Vernon Hills, IL, USA) and transported to the research vessel within 2 h. Prior to sampling, containers, bilge pumps and tubing were washed once with 1% bleach and 0.1 N NaOH, three times with freshwater and once with 100-kDa-filtered seawater. On shipboard the large eukaryotes were removed by filtration through 100 μm Nitex. The filtrate was then concentrated to <500 ml using a 100-kDa tangential flow filter, which captured the unicellular eukaryotes, microbes and virus-like particles. Microbial cells were collected by passing the concentrated sample through 0.45-μm Sterivex filters (Millipore Inc., Billerica, MA, USA) and the filters were stored at −80 °C.

After removing the Sterivex filters, DNA was extracted by standard phenol:chloroform DNA protocols. The DNA was amplified using φ29 polymerase (Monserate Biotech, La Jolla, CA, USA), four to six replicates per site. The replicates were pooled and purified using silica columns (Qiagen Inc., Valencia, CA, USA). The DNA was precipitated with ethanol, re-suspended in water (~300 ng ml^{-1}) and then pyrosequenced at the University of Carolina (Engencore) using the 454 GS-FLX platform.

Metagenomic sequences were uploaded to the MG-RAST server (http://metagenomics.nmpdr.org/metagenomics.cgi) for functional and taxonomic annotation. Sequences were compared to the SEED protein database using BLASTX (Altschul *et al.*, 1990). Sequences with significant similarities (*E*-value <0.00001) were assigned functions based on their closest similarity (Meyer *et al.*, 2008) and were then grouped into metabolic pathways

(subsystems). Also included in the subsequent analyses were metagenomic libraries from four atolls in the Northern LI (Dinsdale *et al.*, 2008b).

Sequence comparison to the virulence factor database
The six metagenomic libraries were compared to the virulence factor database (Chen *et al.*, 2005) (http://www.mgc.ac.cn/VFs/) using BLASTX (*E*-value <0.0001).

Microbial abundances
Direct counts of microbes (Bacteria and Archaea) were made by epifluorescence microscopy. Seawater samples were obtained at each site at the same time of day from 25 cm above the benthos (~10 m depth), then fixed in paraformaldehyde (2% final concentration) within 1 h of collection, filtered onto 0.02-μm anodisc membrane filters (Whatman Inc., Florham Park, NJ, USA) and stained with SYBR Gold (5 × final concentration; Invitrogen, Carlsbad, CA, USA). Microbes were counted in 10 fields selected at random (>200 per sample).

Statistics
Statistical analyses were performed using R (Gentleman and Ihaka, 1996). The non-parametric Mann–Whitney *U*-test, performed using the Wilcox test procedure, was used to assess significant differences between reef sites. Non-metric multidimensional scaling (nMDS) used the isoMDS function with a Euclidean distance matrix. A similarity profile test (SIMPROF) (Clarke *et al.*, 2008) was used to determine whether similarities observed between data sets (metabolic profiles in this study) are significant or likely arose by chance. The test was based on a Bray–Curtis similarity matrix and groupings were examined at the 5% significance level (<0.05) using a maximum of 1000 random permutations of the raw data.

Results and Discussion

Five of the LI have shipwrecks on them resulting in black reefs that are characterized by low coral and CCA cover. As shown in Figure 1, the benthos at these sites appears very dark red or black with increased abundances of turf algae, macroalgae, cyanobacterial mats and/or corallimorphs. The surrounding water is cloudy, with elevated concentrations of particulate organic matter. We surveyed the three largest black reefs and describe their natural history below.

Natural history of the Millennium Atoll black reef
Millennium, formerly Caroline Island, is the most southern atoll of the LI (latitude: −9.955, longitude: −150.210). The reefs on Millennium were characterized by high fish abundances (Barott *et al.*, 2010),
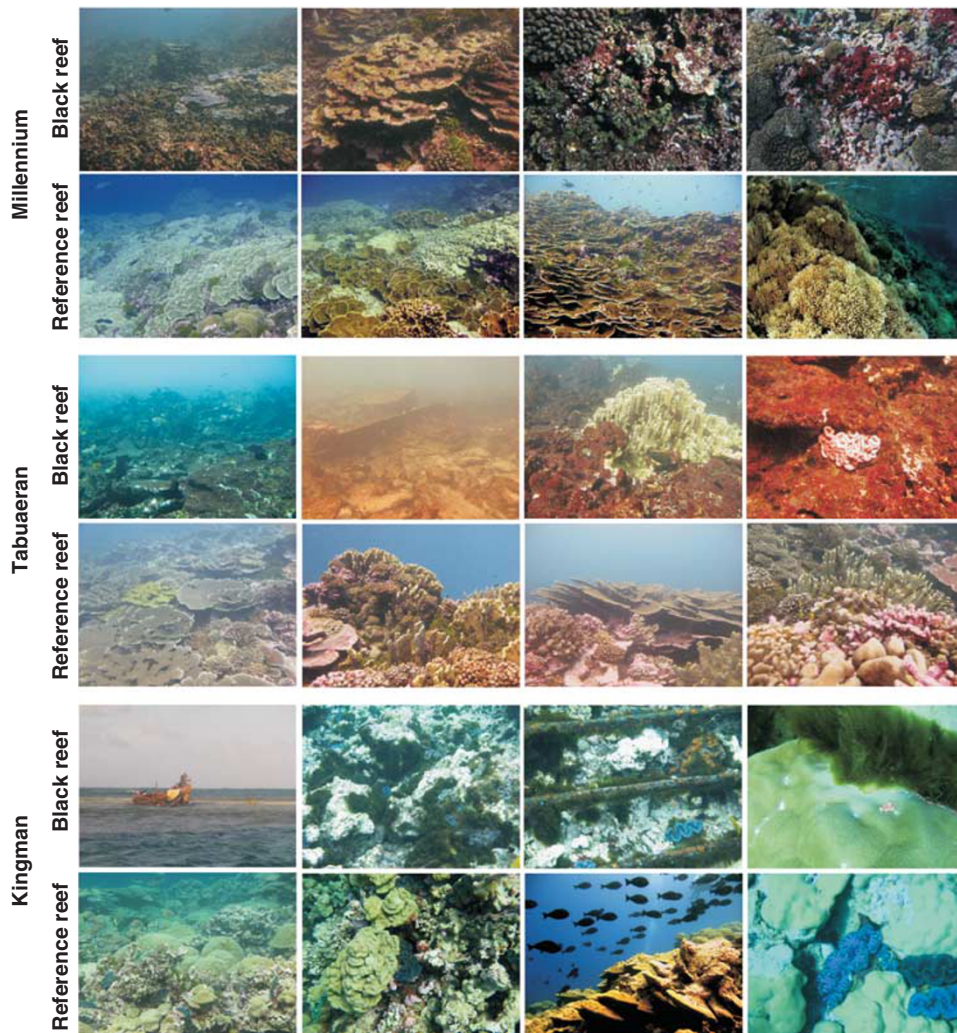
Figure 1 Representative photographs of benthic habitats from black reefs and reference reefs on Millennium (rows 1 and 2), Tabuaeran (rows 3 and 4) and Kingman (rows 5 and 6).

high abundances of stony corals and CCA, and low abundances of benthic algae (Figure 1). The 1993 shipwreck occurred when an 85 ft steel tug ran aground while towing a sailing vessel out of a narrow reef passage. Figure 2a compares the benthic community composition from the black reef with other sites around the atoll. The Millennium black reef extended for 0.75 km² down-current of the shipwreck debris field. Live coral cover was the most abundant benthic functional group on reference sites around Millennium (Figure 2a, mean value, 65.9%, $n = 10$, s.e. ± 4.5) compared with 16.7% ($n = 10$, s.e. ± 4.3) on the black reef. The algal assemblages present on the Millennium black reef are predominately turf algae and cyanobacterial mats.

Natural history of the Tabuaeran black reef
Tabuaeran Atoll, located north of the equator (latitude: 3.880, longitude: −159.320), historically

supported guano mining and copra farming, and is currently inhabited by ∼2500 people. One of ships used in the copra trade ran aground just north of the lagoon channel on the leeward side of the atoll. The black reef associated with this wreck now covers approximately 10% of the surrounding reef (that is, 1/10 of the circumference of Tabuaeran). The spread of the black reef appears to be inhibited only by the two natural barriers created by the strong lagoonal current in the south and the high energy, sand-bottom surf zone in the North (surveyed by Rohwer and Vermeij in 2010). The black reef consisted predominately of dead coral and rubble covered with thick cyanobacterial mats and turf algae (Figure 1). The reefs at the shipwreck site were investigated in August 2005 and November 2010. In 2005, live coral cover was <5% compared with 40.2% ± 14.8 and 28.9% ± 5.1 on leeward reef sites north and south of the wreck, respectively (Figure 2b). In 2010, there was no coral recovery in the sites surveyed in 2005 and the black reef had

extended to the lagoon entrance. Although we do not know the exact date of this shipwreck, it is at least 40 years old (Maragos, JE, personal communication), showing that these wrecks cause persistent reef degradation and represent a long-term threat to LI reefs.
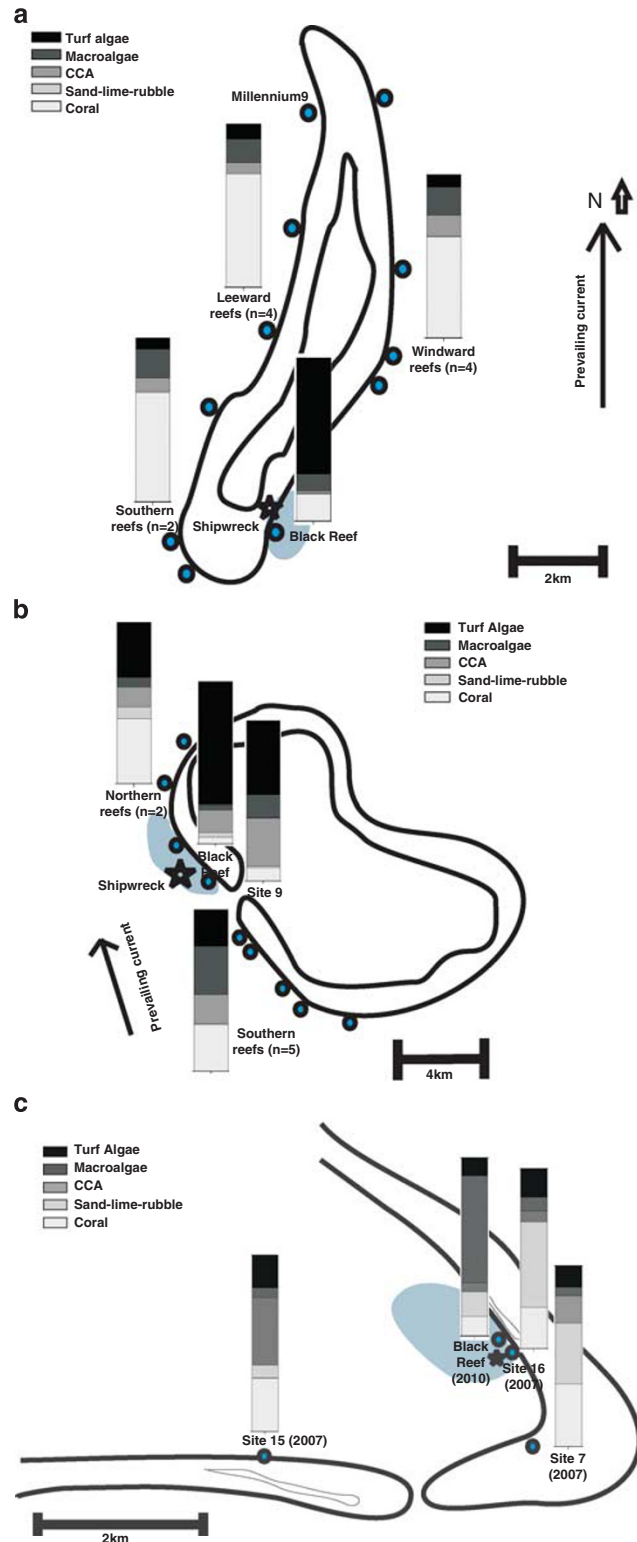
Natural history of the Kingman black reef
Kingman, an atoll located in the northern-most region of the LI (latitude: 6.400, longitude: −162.380), was a National Wildlife Refuge until 2009 when it became a US Marine National Monument. This protected pristine ecosystem shows an inverted biomass pyramid (85% of the total in top predators), high coral recruitment and coral cover, and the second highest coral diversity in the central Pacific (Sandin *et al.*, 2008; Maragos and Williams, 2011). The shipwreck here is a teak-hulled fishing vessel filled with iron-rich compressors, engines and unidentifiable machinery. When first observed in 2007, the hull was located on the fore-reef side of the northeast islet. By November 2010, the wreck had been pushed across the islet and debris was spread across both the fore- and back-reef.

The reef surrounding the Kingman shipwreck has been overgrown by the filamentous green algae *D. tenuissima* (Tsuda RT, personal communication; Figure 1). At the wreck site, *D. tenuissima* covered up to 80% of the benthos, whereas coral plus CCA cover declined to <10% (compared with <15% turf plus macroalgae and ∼50% coral plus CCA in 2007; Figure 2c). *D. tenuissima* overgrowth extended 1.5 km along the reef horizontally and algal overgrowth was observed down the reef slope to ∼35 m (Rohwer and Williams, personal observation, 2010). As *D. tenuissima* was not previously observed in this region, it may be invasive and/or have been introduced by the ship.

Other examples of black reefs in the central Pacific
Similar black reefs have been observed on Canton, Enderbury, McKean, Nikumaroro and Phoenix of the Phoenix Islands (Supplementary Figure 1). The Millennium, Tabuaeran and Kingman black

Figure 2 (**a**) The benthic community composition present on Millennium atoll. The bar graphs represent the percent cover of coral, CCA, macroalgae and turf algae on reefs from Millennium (0.72 m² quadrats per site, $n = 20$). On the black reef, the average percent cover of various benthic organisms was as follows: corals 16.7% ± 4.3, CCA 1.5% ± 0.6, turf algae 71% ± 4.9 and macroalgae 10.6% ± 2.0. For 10 control sites around Millennium, the average percent cover of various benthic organisms was as follows: corals 65.9% ± 7.6, CCA 9.2% ± 2.9, turf algae 7.9% ± 2.3 and macroalgae 16.3% ± 4.5. The photographs depict benthic assemblages characteristic of leeward and windward reefs, and of the black reef. The black arrow indicates the direction of the prevailing current. Water flowed out of the lagoon through the passage where the shipwreck occurred and to the south. (**b**) The benthic community composition present on Tabuaeran. The photographs depict the benthic assemblages characteristic of the northern leeward reefs, site-2 (black reef), site-9 and of the southern leeward reefs on Tabuaeran. The bar graphs represent the percent cover of coral, CCA, macroalgae and turf algae. The black arrow indicates the direction of the prevailing current. (**c**) The benthic community composition for back-reef sites on Kingman Reef. Sites 7, 15 and 16 were measured in August 2007 prior to the influence of shipwreck debris. The black reef site was measured in October 2010. The area of reef estimated to be impacted by shipwreck debris is indicated with light blue shading.

reefs cover relatively large areas; however, we have also observed small versions of the same phenomenon around lesser localized iron sources (for example, lost anchors, steel piping) on Enderbury (Supplementary Figure 2A) and Jarvis (Supplementary Figures 2B and C).

Summary of LI black reefs
The natural history of these black reefs shows that they: (1) are always congruent with shipwrecks or a point source of iron; (2) can develop quickly after a shipwreck (<3 years in the case of Kingman); (3) persist for decades (for example, Tabuaeran and Millennium); (4) most consistently and abundantly include a source of iron (for example, not copper paint, petroleum products, etc) and (5) have similar biotic composition (turf algae, macroalgae, cyanobacterial mats and corallimorphs), with variation in the relative abundances of these groups. This variation may represent stages of succession or differences in the biogeography of specific taxa.

The LI occur in a high-nutrient, low-chlorophyll region of the ocean. Paradoxically, high-nutrient, low-chlorophyll regions have low primary production despite higher concentrations of phosphate and nitrogen. Martin *et al.* (1994) resolved this apparent paradox by showing that high-nutrient, low-chlorophyll waters are iron-limited; addition of iron leads to a rapid increase in primary production. Figure 3a depicts the estimated iron concentrations for the world's oceans based on iron deposition. Given this regional background and the observation that iron points sources are associated with all black reef sites, we hypothesized that iron supplementation was supporting the large-scale algal overgrowth of corals on black reefs.

Comparison of iron concentrations in mixed algal assemblages
To investigate whether there was more bioavailable iron on the black reef, the incorporation of elemental iron into algal tissue was measured using inductively coupled plasma (ICP-OS). Iron concentrations measured in mixed algal assemblages from the Millennium black reef were six times higher than in assemblages from reference sites (Figure 3b and Supplementary Table 1; median values 633 µmol Fe:mol C at the black reef versus 105 µmol Fe:mol C at Millennium reference sites and 83 µmol Fe:mol C at other LI control sites). The iron concentrations for the black reef were significantly different from reference sites on Millennium (Mann–Whitney U-test, two-tailed, $n = 10$, P-value $= 0.009$) and from reference sites on other LI reefs ($n = 11$, P-value $= 0.018$).

For these comparisons, mixed algal species (composed mainly of red algae and CCA) were isolated from coral rubble (Supplementary Table 1).

Consequently, the proportion of different algal species present in each sample could not be quantified. Although some deviations in iron concentration may be attributable to differences in algal species composition, the large differences observed in this study likely reflect environmental iron availability. The iron concentrations of mixed algae collected from LI reference sites were comparable to those found in macro- and turf algae from Davies Reef, GBR (Supplementary Table 1; Entsch *et al.*, 1983). Although the iron concentrations in filamentous cyanobacteria on Davies Reef were 2- to 4-fold higher than in species of red and green macroalgae, the iron concentrations of mixed algal assemblages from the Millennium black reef were three-fold higher than the Davies reef cyanobacteria and nearly an order of magnitude higher than the average Davies Reef macroalgae. In addition to iron, 13 other elements (magnesium, zinc, manganese, selenium, nickel, lead, chromium, cadmium, copper, mercury, arsenic, sulfur and strontium) were measured in samples collected from the black reef using ICP. None of these metals were enriched at the shipwreck sites as compared with reference sites (data not shown).

The increased iron concentrations in algal assemblages adjacent to the shipwreck debris indicate an increase in bioavailable iron. While the form of iron released from the corroding ship (for example, $Fe(OH)_3$) may not be immediately available for uptake by algae, ferric iron can be solubilized through the complexation of high-affinity organic ligands, which substantially increases its bioavailability (Rue and Bruland, 1995; Johnson *et al.*, 1997). Although the complexities of iron biogeochemistry are largely unknown (Tortell *et al.*, 1999), there is compelling evidence that the insoluble iron pool is rapidly complexed, thus increasing its bioavailability to microbes and eukaryotic algae (Rue and Bruland, 1997; Granger and Price, 1999; Amin *et al.*, 2009). The specific mechanism whereby the iron associated with the shipwreck debris is becoming available to the benthic algae has not been determined; nevertheless it is evident that iron is being recycled in marine environments and is accumulating in the algae around the Millennium black reef.

The effects of iron enrichment on coral health
The effects of iron enrichment on coral and coral/algal communities were investigated using *P. meandrina* corals collected from a healthy reference site on Tabuaeran maintained in mesocosms. Corals were incubated for 5 days in the presence or absence of ferric chloride ($FeCl_3$) and in the presence or absence of algal-covered rubble collected from the black reef on Tabuaeran (Figure 4). Corals incubated with both iron and rubble communities showed the greatest mortality (Figures 4a and b, 50% dead, 20% dying), followed by the coral plus rubble treatment

a

b
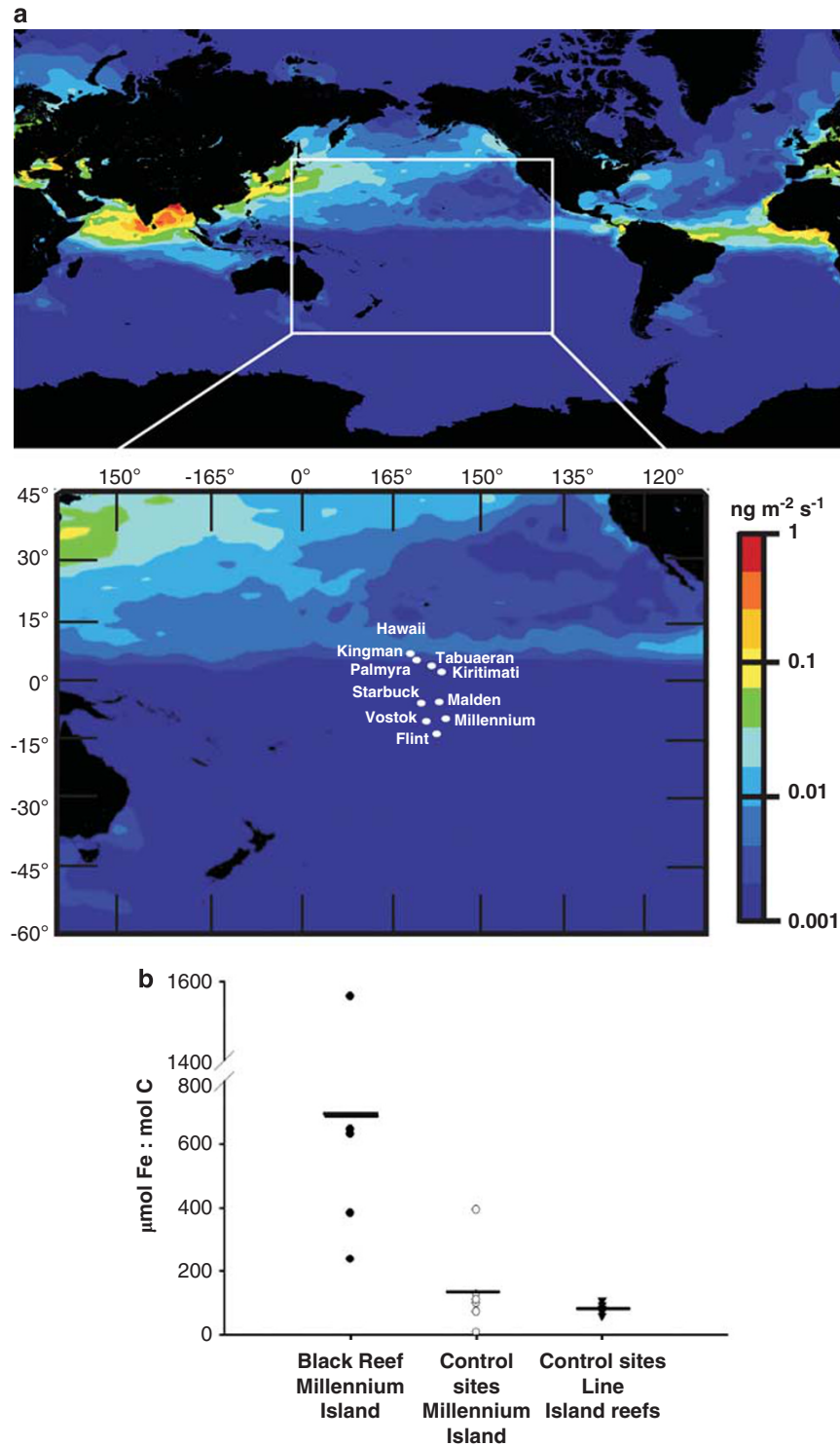
Figure 3 Regional and local iron concentrations. (**a**) Iron concentrations across the world with an emphasis on the central Pacific (data for oceanic iron concentrations were taken from Behrenfeld *et al.*, 2009). The colored bar estimates iron deposition ($ng\,m^{-2}\,s^{-1}$). (**b**) The elemental stoichiometry of iron incorporated into the tissues of benthic organisms from the black reef, control sites on Millennium and from other LI reefs. Iron and carbon concentrations were measured using ICP-OS and EA, respectively. The horizontal line represents the mean of each data set.

(20% dead, 30% dying). Enhanced microbial activity was previously linked to mortality of corals in the presence of macroalgae (Smith *et al.*, 2006). To determine whether the coral mortality observed here was mediated by microbes, the same treatments were conducted with antibiotics added. Addition of ampicillin significantly reduced coral death in the presence of iron and rubble (10% dead, 10% dying;
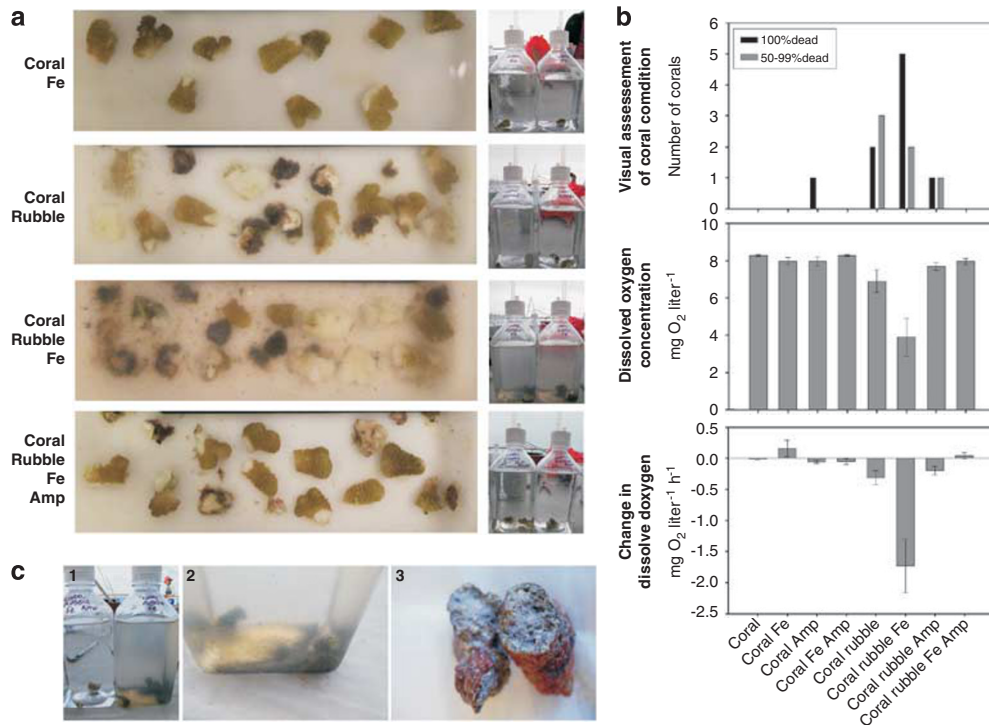
Figure 4 Mesocosm experiments measuring the response of corals and algal-covered rubble to iron enrichment. (**a**) *P. meandrina* corals were treated with $1 \mu M$ ferric iron ($FeCl_3$) with and without the presence of black reef algae. The same treatments were conducted plus $50 \mu g \, ml^{-1}$ ampicillin. Coral fragments and mesocosms were photo documented after 5 days (4 of 8 treatments shown, $n = 10$). (**b**) Coral condition was visually assessed (top panel). The concentration of DO (mg $O_2 \, l^{-1}$) in the aquarium water was measured at the completion of the experiment (middle panel). The rate of change in DO (mg $O_2 \, l^{-1} \, h^{-1}$) was also measured (bottom panel). Fe, ferric iron; Amp, ampicillin. (**c**) Photographs of iron-enriched mesocosms depicting water turbidity (1) and the formation of black particulate material characteristic of the black reef (2). Cross-section of algal-covered rubble collected from the Millennium black reef (3).

Figure 4b; Mann–Whitney *U*-test, two-tailed, $n = 20$, *P*-value $= 0.007$).

The water in the iron-enriched mesocosms turned cloudy, suggesting possible elevated growth rates of phytoplankton and heterotrophic microbes (Bacteria and Archaea) (Figure 4c). Iron availability is known to stimulate heterotrophic microbial production indirectly through release of phytoplankton-derived dissolved organic matter (Obernosterer *et al.*, 2008). Therefore, DO consumption was used to estimate respiration by heterotrophic microbes in these mesocosms. After 5 days of incubation with added iron plus rubble, the concentration of DO was reduced from 7.9 ± 0.2 mg $O_2 \, l^{-1}$ in controls to 3.9 ± 1.1 mg $O_2 \, l^{-1}$ (Figure 4b), whereas DO consumption increased from 0.06 ± 0.05 to 1.7 ± 0.2 mg $O_2 \, l^{-1} \, h^{-1}$.

Metagenomic analysis of the microbial community associated with a black reef
Metagenomic sequencing was used to compare the microbial community associated with the Millennium black reef with that on near-pristine reefs in the region, including a healthy reference site on Millennium. In this study, six libraries with a total of 237 282 sequences were obtained from six sites at four southern LI atolls: Millennium black reef; reference sites on Millennium, Flint (2), Starbuck

and Vostok (Table 1). Data for four atolls in the northern LI (Dinsdale *et al.*, 2008b) were also included in the analysis. Metabolic profiles were created for each site based on the relative abundances of sequences similar to known sequences assigned to 25 SEED metabolic subsystems. The 10 subsystem profiles were compared simultaneously by nMDS. The Millennium black reef site grouped with Kiritimati, Kingman and Palmyra, and showed closest similarity to the degraded Kiritimati reef, on a scatter-plot of the first two nMDS dimensions (Figure 5). A similarity profile test (SIMPROF) determined three significant groupings within the data set, mirroring the patterns visualized by the nMDS plot (Supplementary Figure 3). The metabolic profile of the microbial community from the Millennium black reef differed significantly from the Millennium control site. Pathways involved in heterotrophic metabolism were 10-fold higher on the black reef (data not shown). Although sequence similarities to genes do not represent levels of gene expression, metagenomes have been shown to be strong predictors of the biogeochemical conditions driving the microbial community (Dinsdale *et al.*, 2008a).

Phylogenetic characterization of the microbial community supported the metabolic profiles. The black reef had proportionally more heterotrophic

Table 1 Total number of sequences from each metagenomic library

Library name	Average sequence length	Total no. sequences	Total library size (bp)	Total no. bacterial similarities	Total no. subsystem similarities
Millen. black reef	234.7	46 782	10 979 616	14 053	9826
Reference sites					
Millen. 9	250.2	7340	1 836 674	980	782
Flint 2	240.0	21 441	5 145 171	1620	3170
Flint 5	243.4	32 832	7 990 372	1731	578
Starbuck 7	240.3	39 335	9 453 923	4989	5865
Vostok 10	233.7	89 552	20 924 215	14 685	4308
Total	240.4	237 282	56 329 971	38 058	24 529

Abbreviation: Millen., Millennium.
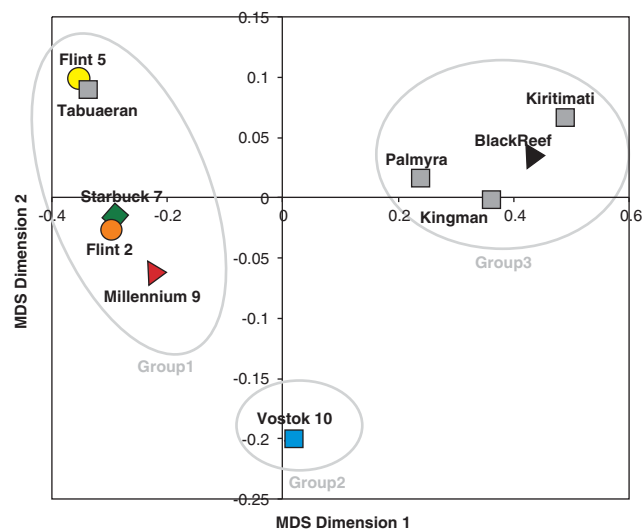
Figure 5 Metabolic profile for the microbial community inhabiting the black reef compared with nine other coral reefs. The scatter-plot depicts the first two dimensions of the nMDS analysis. The 10 microbial metagenomes used for this analysis include six southern and four northern LI coral reefs (Dinsdale *et al.*, 2008b). Metagenomic libraries collected from northern LI reefs are depicted with gray squares. Metagenomic libraries collected from the same atoll are depicted with the same symbol (for example, circles for Flint and triangles for Millennium). The three grouping shown were determined by a similarity profile test ($P \leqslant 0.05$).

Table 2 Abundances of virulence factor genes from microbes associated with Southern LI reefs

Site	Hits to virulence database	Normalized to total bp ($\times 10^5$)	Iron-related virulence genes
Millen. black reef	1068	9.7	126
Millen. 9	51	2.8	3
Flint 2	230	4.5	4
Flint 5	18	0.2	0
Starbuck 7	341	3.6	15
Vostok 10	244	1.2	3

Abbreviations: Millen., Millennium; LI, Line Islands.

in potential opportunistic coral pathogens (Anderson, 1998; Harvell *et al.*, 1999).

Virulence genes are overrepresented in the black reef-associated microbial community
Greater abundances of pathogen-like microbes and virulence factor genes were observed on degraded reefs (Dinsdale *et al.*, 2008b), as well as in stressed corals (Thurber *et al.*, 2009). To determine whether virulence genes increased on black reefs, the six metagenomic libraries were compared to the virulence factor database (VFDB) (Chen *et al.*, 2005). The black reef sequences included 1068 significant similarities (Table 2, BLASTx, *E*-value < 0.0001), which when normalized for library size represented a 2- to 10-fold greater abundance of virulence genes than the other sites. More telling, virulence genes related to iron comprised 12% of the total virulence genes (126) on the black reef compared with 0–5% for the other sites (Table 2). These results suggest that iron enrichment may select for a subset of potentially virulent microbes that can better scavenge and import iron.

Implications of coral to algal phase shifts for the associated microbial community
These changes in the Millennium black reef microbial community suggest an influence by the benthic algal overgrowth, in addition to the iron enrichment.

microbes than the reference sites on Millennium, Flint (two sites) and Starbuck (Supplementary Figure 4). Additionally, the microbial abundances were three times higher on the Millennium black reef ($20.5 \times 10^5 \pm 4.0 \times 10^5$ cells ml^{-1}, $n = 3$) as compared with all other Millennium reef sites ($7.4 \times 10^5 \pm 0.7 \times 10^5$ cells ml^{-1}, $n = 23$). The differences in microbial abundances were statistically significant (Mann–Whitney *U*-test, two-tailed, *P*-value = 0.016). These changes in the microbial community are similar to previous observations by Dinsdale *et al.* (2008b) where the degraded, algae-cover reefs of Kiritimati Atoll were dominated by heterotrophic, pathogen-like microbes. One implication of this community shift is an increase

Here the proposed mechanism is the stimulation of heterotrophic microbes by the dissolved organic carbon released by the benthic algae, which in turn leads to asphyxiation of the coral (Smith et al., 2006). Turf algae on coral reefs can exude dissolved organic carbon at rates of 12.2 ± 2.1 mg C m^{-2} h^{-1} (Haas et al., 2010b), whereas corals are net consumers of dissolved organic carbon (Haas et al., 2010a). This suggests that the composition of the benthic community can influence the availability of organic matter, thereby providing a possible mechanism for the observed shifts in the microbial community associated with the black reef. The increase in potential pathogens observed on the black reef might be contributing to coral death and/or inhibiting coral recovery (Vermeij et al., 2009), thus further increasing the available dissolved organic carbon. Furthermore, the mesocosm experiments demonstrated that black reef rubble algae were lethal to corals even without iron enrichment, thus predicting long-term consequences of the phase shift after removal of the iron.

Caveats
Owing to the remote nature of these sites, we do not have complete data sets from all of the black reefs. Future studies need to include ICP-OS analysis of the algae and metagenomic analysis of the microbial communities from the other sites whose black reefs are of different ages. Current observations indicate a benthic succession following a shipwreck perturbation. Corallimorphs are common around the 1991 Palmyra shipwreck, but only present on the leading edges of the younger black reefs on Kingman and Millennium, and absent from the >40-year-old Tabuaeran black reef.

One drawback of our experimental design was that we could not compare iron concentrations of the same algal species on black reefs and reference reefs. Neither the dominant turf-cyanobacterial assemblages on Millennium/Tabuaeran nor the green alga prevalent on Kingman were visibly present at reference sites. To address this, algae should be transplanted from reference sites to black reefs and their iron concentrations measured subsequently. Dissolved iron concentrations in the water column surrounding the wrecks should also be measured.

While sunken ships on some tropical reef flats may promote coral growth by providing a substratum for settlement, these artificial reefs are successful in regions, such as Hawaii or the Caribbean, that are not iron-limited and where algal growth is controlled by other nutrients.

Microbial ratchet
One enigmatic observation is the large distances that the black reefs extend from the shipwrecks. As iron transport through the water would lead to mixing and dilution, we hypothesize that a re-mineralizing microbial–algal mat uses a ratchet-like mechanism to crawl down the reef. In this model, the microbial–algal mats on the point source scavenge iron, overwhelming the corals and CCA in the immediate area. As they expand outward, they carry iron within them that, upon their death, becomes locally available. This would enable the mat to extend farther still from the original shipwreck site and retain the higher concentration of iron. In any case, there is a fascinating sediment–microbe–algae–iron re-mineralization story on the black reefs awaiting future studies.

Conservation implications
Ecological disturbances that support algal growth on reefs can reach critical thresholds resulting in a shift to an alternative stable state (Hatcher, 1984; Knowlton, 1992). This new state is then maintained by self-reinforcing feedback mechanisms (Nystrom et al., 2008). On coral reefs, it has been posited that the shift to an algal-dominated state could be irreversible even after the disturbance is resolved because a feedback may exist if the algae are detrimental to coral recovery (Knowlton, 1992; Norstrom et al., 2009). Possible mechanisms for this include algal influence on water clarity, nutrient availability, microbial activity and allelopathy (Abram et al., 2004; Smith et al., 2006; Rasher and Hay, 2010).

We have shown here that some of the most pristine coral reefs in the world are particularly sensitive to shipwrecks and the associated iron. These findings also have significant implications for the unpredictable problems that may result from iron fertilization projects in the ocean. Coral recovery requires substantial reproductive success followed by prolific settlement and growth (Done, 1992). For the LI black reefs, recovery is promising after remediation of shipwreck sites because these remote reefs are spared many anthropogenic impacts, such as overfishing and pollution, and because high densities of coral cover nearby increase the likelihood of repopulation by scleractinian corals (Schroeder et al., 2008). In summary, we would like to emphasize the importance of removing shipwrecks from coral reefs. These near-pristine reefs in the central Pacific should be preserved at all costs as they represent our last glimpse of one of the planet's most remarkable environments.

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Author contributions

The paper was written by LWK and FLR. The iron measurements and metagenomic analyses were completed by LWK. The sample collection and field experiments were completed by KLB, ED, AMF, MJAV, ES and FLR. The benthic characterizations were completed by JES and GJW. SAS and DO characterized the black reefs on Millennium, Starbuck, Tabuaeran and the Phoenix Islands. BN constructed the iron map using GIS and helped with the bioinformatics. DW completed the statistical calculations and provided valuable computational support to LWK. The SIMPROF analysis of the metabolic sequence data was completed by GJW. All of the authors offered helpful comments to the paper.

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